TRICHORZIANINS ACTIVITY ON MYCELIAL GROWTH OF *SCLEROTIUM CEPIVORUM* UNDER LABORATORY CONDITIONS *IN VITRO*

ALEJANDRO CORREA[1], MARIE-FRANCE ROQUEBERT[2] and LINA BETTUCCI[1]

[1] Sección Micologia, Facultad de Ciencias, Universidad de la República. T. Narvaja 1674, CP 11200, Montevideo, Uruguay.
[2] Laboratoire de Cryptogamie, MNHN, Paris.

ABSTRACT Peptidic secondary metabolites, trichorzianins, obtained from *Trichoderma harzianum* inhibited mycelial growth of *Sclerotium cepivorum*. Biossays showed the inhibitory activity of the crude natural trichorzianin mixture causing up to 100 % at 500 $\mu g.ml^{-1}$. Silica gel chromatography resolved this mixture in two groups, trichorzianins TA neutral and trichorzianins TB acidic that had different inhibitory activities on mycelial growth of *S. cepivorum*. The mixture of trichorzianins had higher activity than TA and TB separately, suggesting a synergistic effect on the inhibition of mycelial growth.

KEY WORDS: Inhibitory activity, peptide antibiotics, *Sclerotium cepivorum*, *Trichoderma harzianum*, trichorzianins.

RÉSUMÉ — Des métabolites secondaires peptidiques, les Trichorzianines, produits par *T. harzianum*, inhibent la croissance mycélienne de *Sclerotium cepivorum*. Des essais *in vitro* montrent une activité inhibitrice d'un extrait brut (mélange de Trichorzianines) allant jusqu'à 100 % à 500 $\mu g.ml^{-1}$. La chromatographie sur gel de silice sépare le mélange en 2 fractions: Trichorzianines A neutres et Trichorzianines B acides, qui ont des activités inhibitrices différentes sur la croissance de *S. cepivorum*. Le mélange TA et TB est plus actif que chaque fraction utilisée séparément, ce qui suggère une synergie d'action.

MOTS-CLEFS: Inhibition de croissance, antibiotiques peptidiques, trichorzianines, *Trichoderma harzianum*, *Sclerotium cepivorum*.

INTRODUCTION

Three categories of antibiotics compounds produced by *Trichoderma harzianum* Rifai (and other *Trichoderma* species) can be recognized: "volatiles", e.g. 6-pentyl--pyrone and most of the isocyanide class of compounds; "leachables" materials with some solubility in water; and "peptaibol", which consists of hydrophobic peptides (Ghisalberti & Sivasithamparam, 1991). Trichorzianins are a mixture of

nonadecapeptides of the peptaibol class produced by *T. harzianum* isolated and characterized by El Hajji *et al.* (1987). They are linear peptides with an acetylated N terminal residue and a C terminal amino alcohol which interact with lipidic membranes and modify their permeability (El Hajji *et al.*, 1989). Two classes of trichorzianins termed TA and TB were characterized: TA were a microheterogeneous mixture of neutral peptides and TB the acidic analogues due to the replacement of a glutamine at position 18 in the sequence by a glutamic acid (Bodo *et al.*, 1985; El Hajji, 1987).

Sclerotium cepivorum Berk. is a typical root-infecting fungus confined to the genus *Allium*, producing white rot disease (Esler & Coley-Smith, 1984). Several strains of *Trichoderma harzianum* were found to be active inhibitors on mycelial growth and sclerotia formation of *S. cepivorum*. Dual culture and cellophane membrane technique were used to evidence this antagonistic activity (Dennis & Webster, 1971; Jackson *et al.*, 1991).

This work was carried out in order to evaluate the potential inhibitory activity against *S.* cepivorum of trichorzianins produced by *T. harzianum*.

MATERIALS AND METHODS

Fungal strains

Trichoderma harzianum (MVHC 6063) was used for production of trichorzianins and *Sclerotium cepivorum* (MNHN 5138, Laboratoire de Cryptogamie, Paris) was the plant pathogen used in this study. Each fungus was subcultured on 2% malt agar (MA) and grown at 24°C in the dark. The strains were preserved on 2% MA slopes at 5°C.

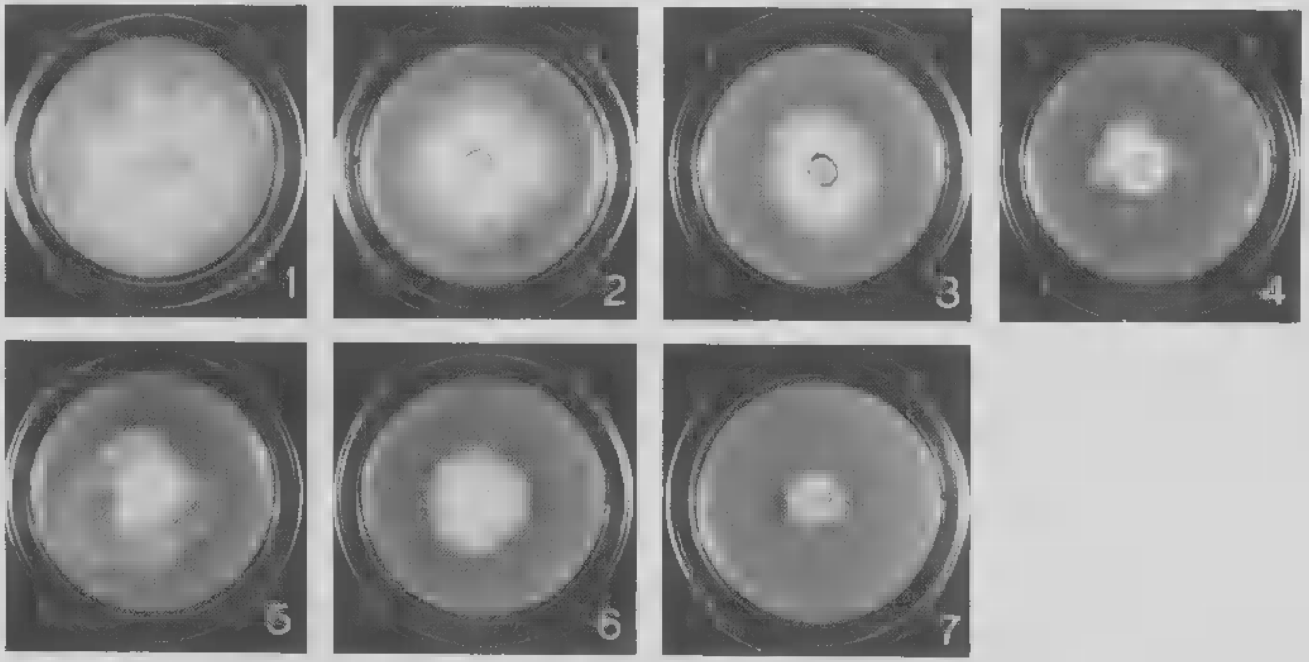
Extraction of trichorzianins

Trichoderma was inoculated using 1 ml of spores suspension into 200 ml of synthetic medium in 1.0 l Roux flasks. The synthetic media was composed of the following: 5.0 g glucose; 0.8 g KH_2PO_4; 0.7 g KNO_3; 0.2 g $CaHPO_4$; 0.5 g $MgSO_4$; 10 mg $MnSO_4$; 10 mg $ZnSO_4$; 5 mg $CuSO_4$; 1 mg $FeSO_4$; in 1.0 l distilled water pH 6. Stationary culture (60 flasks) were incubated at 24°C until sporulation.

Extraction of trichorzianins was performed according to the method of Rebuffat *et al.* (1991) previously described by Correa *et al.* (1995).

Bioassay

The different fractions obtained were tested for their antagonistic activity against the mycelial growth of *S.cepivorum*. A methanol solution of each fraction was



Figures 1-7. Peptides growth inhibition of *S. cepivorum* after 120 h. 1: *S. cepivorum* control growing on MA medium. 2-4: mixture of peptides at different concentration (25, 50, and 100 $\mu g.ml^{-1}$). 5: *S. cepivorum* control growing on MA-methanol medium. 6: mycelial fraction. 7: mixture of peptides at 500 $\mu g.ml^{-1}$.

Figures 1-7. Inhibition de croissance de *S. cepivorum* par les peptides, après 120 h de culture. 1: Témoin sur Malt-Agar. 2-4: mélange de peptides aux concentrations de 25, 50 et 100 $\mu g.ml^{-1}$. 5: Témoin sur milieu Malt-Agar Méthanol. 6: Métabolites extraits du mycélium. 7: Mélange de peptides à 500 $\mu g.ml^{-1}$.

mixed with 2% liquid MA (45-50°C) and 2.5 ml were poured in Petri dishes (5 cm diameter). A 6 mm disk from the edge of a growing colony was inoculated in the center of the dish. Four replicates of each were performed. The inhibitory activity of the peptide mixture was analyzed at concentrations of 25, 50, 100 and 500 $\mu g.ml^{-1}$ of medium and the trichorzianins A and B at 100 $\mu g.ml^{-1}$. A set of Petri dishes with each respective methanol concentration was mixed with malt-agar and inoculated with *S. cepivorum* as controls. The diameter of the colonies was measured and the percentages of growth inhibition at 72 h were calculated as follows: 100-(dt.100/DT), where dt is the diameter of the treated colony and DT is the diameter of the control. Differences between the inhibitory activity of each *T. harzianum* extract on the *S. cepivorum* mycelium were determined by means of ANOVA (Service des Etudes Statistiques, Institute technique de Céréales et Fourrages, STAT-ITCF)

RESULTS

The hexane soluble fraction from mycelia and broth did not affect mycelial growth of *S. cepivorum*. The hexane insoluble fraction extracted from mycelium inhibited mycelial growth of *S. cepivorum* 33% ($P < 0.05$).

The hexane insoluble fraction from broth was fractionated in order to get the mixture of trichorzianins. The activity of this trichorzianins mixture was high, causing up to 100 % growth inhibition at a concentration of 500 $\mu g.ml^{-1}$, after 72 h of incubation ($P < 0.05$, Figs. 1-7). Observations made at 120 h showed that this concentration allowed mycelial growth only upwards but never on the culture medium.

This mixture of Trichorzianins was, in turn, fractionated by silica gel chromatography and resulted in two groups of trichorzianins (TA and TB). Trichorzianins TA at 100 $\mu g.ml^{-1}$ inhibited mycelial growth 75%. The same concentration of trichorzianins B inhibited growth only 40% (Table I, Fig. 8).

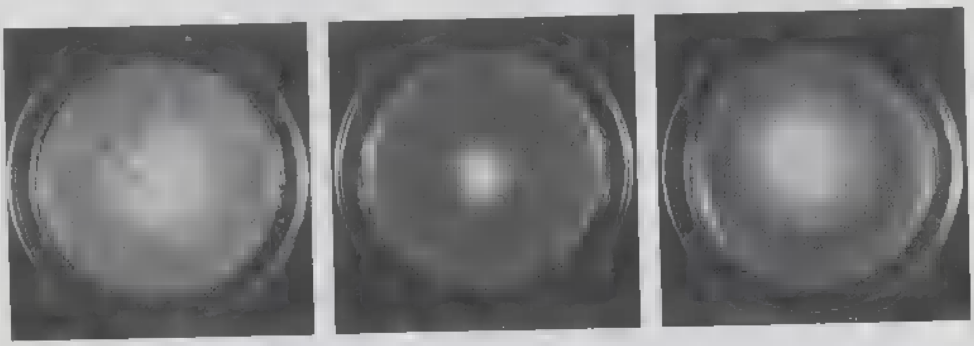


Figure 8. Trichorzianins A and B growth inhibition of *S. cepivorum* after 96 h. Left: *S. cepivorum* growing in fresh media, center: with TA (100 $\mu g.ml^{-1}$), right: with TB (100 $\mu g.ml^{-1}$).

Figure 8. Inhibition de croissance de *S. cepivorum* après 96 h de culture. Gauche: Témoin sur milieu frais. Centre: en présence de 100 $\mu g.ml^{-1}$ de TA. Droite: en présence de 100 $\mu g.ml^{-1}$ de TB.

Products	Concentration ($mg.ml^{-1}$)	GI% (72h)*
Mycelium fraction	$2x10^3$	33
Mixture of trichorzianins	25	36
	50	51
	100	92
	500	100
Trichorzianins A	100	75
Trichorzianins B	100	40

* All the values of GI significantly differed ($P<0.05$) with the controls.

Table 1. Growth inhibitory activity in percentage (GI%) of the different products of the fractioning. All the values of GI significantly differed ($P<0.05$) with the controls.

Tableau 1. Pourcentages d'activité inhibitrice des différentes fractions (GI%).

DISCUSSION

Natural trichorzianins mixture had the highest inhibitory activity at the highest concentration analysed. The mixture of trichorzianins had higher activity than TA and TB separately, suggesting a synergistic effect on the inhibition of mycelial growth. Trichorzianin TA has lower polarity than trichorzianin TB. It was shown that in *Dictyostelium discoideum* amoeba and synthetic membranes (liposomes) trichorzianins A produced a higher membrane permeability than trichorzianins B suggesting a plasma membrane alteration (El Hajji *et al.*, 1989). Therefore it is probable that this higher inhibitory activity of TA on the *S. cepivorum* mycelium growth could be related to more perturbation induced on the plasma membrane than TB.

There are several evidences for the plasma membrane being the locus for perceiving a stimulus for a change in the directional growth (Smith, 1990).

The mycelial fraction had some significant inhibitory activity on *S. cepivorum* conversely to which was observed with *S. rolfsii*. It is probable that both species have different sensibility for these metabolites at the same concentration. The effect of trichorzianins is initially the same on both fungi, reducing the mycelial growth rate. However, the aggregated mycelial (strands) developed by *S. rolfsii* could be consider as an escaper strategy which *S. cepivorum is unable to display*. Thus, in *S. rolfsii* the inhibitory activity was also related to mechanisms implied in growth pattern while in *S. cepivorum* was only fungistatic because mycelial growth was limited but its growth pattern was not modified (Correa, 1994; Correa *et* al., 1995).

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